Forced degradation study of quinapril by UPLC-DAD and UPLC/MS/MS: Identification of by-products and development of degradation kinetics.

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Abstract

Quinapril undergoes a significant degradation in the solid state, especially in the presence of humidity, temperature and pharmaceutical excipients. Since dissolution increases the degradation, hydrolytic reactions are among the most common processes involved in drug degradation. Improving the knowledge regarding drug stability, especially concerning the critical factors that can influence the stability of the active substance in solutions, such as the temperature, the pH and the concentration of catalytic species usually acids or bases is essential for pharmaceutical use; the aim of this study was therefore to develop a new chromatographic method for rapidly and accurately assess the chemical stability of pharmaceutical dosage in acidic, neutral and alkaline media at 80°C according to the ICH guidelines. Ultra High Performance Liquid Chromatography (UPLC) coupled to electrospray ionization tandem mass spectrometry was used for the rapid and simultaneous analysis of quinapril and its by-products. Separation was achieved using a BEH C18 column and a mixture of acetonitrile - ammonium hydrogencarbonate buffer (pH 8.2; 10 mM) (65:35, v/v) with a flow rate of 0.4 mL/min as a mobile phase. This method allowed the drug by-products profiling, identification, structure elucidation and quantitative determination of by-products under stress conditions. The developed method also provides the determination of the kinetic rate constants for the degradation of quinapril and the formation of its major by-products. Kinetic study and the structure elucidation of by-products allow the development of a complete model including degradation pathway observed under all tested conditions.

Keywords: Quinapril, Stress testing, ICH guideline (Q1A R2), UPLC-MS/MS, Degradation products, Degradation pathway.

Introduction

Quinapril (3S)-2-[(2S)-2-[[(1S)-1-(Ethoxycarbonyl)-3-phenylpropyl]amino]-1-oxopropyl]-1,2,3,4-tetrahydro-3-isoquinolinecarboxylic acid hydrochloride [1-2] is a nonpeptide, nonsulfhydryl angiotensin converting enzyme inhibitor (ACEI) belonging to the third class of ACEI [2-4]. It is used for the treatment of cardiovascular diseases such as hypertension and congestive heart failure, either alone or in conjunction with other drugs [5-6]. Quinapril is an oral prodrug, yielding via metabolism a free active diacid compound, quinaprilat [4-7], which acts as an ACEI [8][9].

Previous studies showed that, like many dipeptide ACEI such as lisinopril [10], moexipril [11-13], enalapril

maleate [14], benazepril [15] and ramipril [16], quinapril hydrochloride is unstable in solid phase (in pharmaceutical dosage), especially in the presence of humidity, temperature, and pharmaceutical excipients [17-18]. The influence of these factors on the stability of quinapril and the pathway characterizing its degradation in the solid phase were investigated by Beata Staniz [17-18]. Analysis of the degradation of quinapril in the solid-state showed that Quinaprilat (hydrolysis product), the diketopiperazine ester (cyclization product) and the diketopiperazine acid (cyclization and hydrolysis product) were the main products [17-18].

Although a significant degradation occurs in the solid state, particularly in amorphous systems, the rate of drug degradation is faster in solution [19].

Hydrolytic reactions are among the most common processes for drug degradation. Improving the knowledge regarding the critical factors that can influence the stability of the active substance in solution, such as temperature, pH and concentration of catalytic species usually acids or bases [20] are of major importance in pharmaceutical development, which include degradation studies in acidic, neutral and alkaline media. In fact, drug impurity profiling, identification, structure elucidation and quantitative determination of impurities and degradation products in bulk drug materials and pharmaceutical formulations is one of the most important fields of activities in modern pharmaceutical analysis. The reason for the increasing importance of this area is that unidentified, potentially toxic impurities are health hazards. Therefore, in order to increase the safety of drug therapy, impurities should be identified and determined by selective methods [20-22].

In the literature, there is no reported work on the chemical stability of quinapril under stress conditions. Further studies are therefore needed to characterize the by-products formed by quinapril degradation under acidic, neutral and alkaline media. According to the international conference on harmonization (ICH), accelerated stability studies have to be carried out, according to the stability test guideline Q1A (R2) [23] to establish its inherent stability characteristics, leading to the development of a separation method for the degradation products and hence to support the stability-indicating nature of the method.

Many analytical methods have been reported in the literature for the analysis of quinapril such as gas chromatography with negative-ion chemical ionization mass spectrometry (MS) or electron-capture detection [24], capillary electrophoresis [25], high performance liquid chromatography with fluorescence [24], UV [2] or radiochemical detection [26] as well as electrospray ionization (HPLC/ESI) [2], and MALDI-TOF MS[27]. The considered techniques include also UV spectroscopy [28], square wave voltammetry [29], ultraperformance liquid chromatography tandem mass spectrometry (UPLC–MS/MS) [28], ultra-thin-layer chromatography mass spectrometry and thin-layer chromatography mass spectrometry [30]. However, to our knowledge, on the one

hand no study demonstrates the ability of ultra-performance liquid chromatography (UPLC) to resolve the separation of quinapril and its degradation products, and on the other hand the identification of the by-products has not been previously investigated. In order to better understand the mechanism of quinapril degradation, a chromatographic method involving diode array detector (DAD) and tandem mass spectrometry (MS-MS) has been therefore developed in this study. Degradation of quinapril according to the ICH guidelines has been performed and a complete mechanism for the quinapril degradation under basic, neutral and acidic conditions has been proposed. Since no data on kinetics of quinapril degradation could be found in the published literature, the purpose of this paper was also to present kinetic studies of disappearance and appearance of quinapril and major degradation products, respectively.

2. Experimental conditions

2.1. Chemical standards and solutions

Quinapril was provided by the National laboratory of control of drugs and screening dopage (Tunisia). All mobile phase were prepared from reagent-grade chemicals and purified water (UPW) delivered by a Millipore system (MilliQ Elga, France). Acetonitrile (ACN) for UPLC-DAD and UPLC-MS/MS was purchased from Fisher Chemicals (HPLC grade, Loughborough, Leicestershire, UK) and JT Baker (LC-MS grade, United States), respectively. Aqueous solution of ammonium hydrogenocarbonate (Prolabo, Paris, France), (10 mM) was adjusted at pH = 8 ± 0.1 with a 911 pH meter (Knick, Germany).

The mobile phases were filtered through a 0.2 µm cellulose acetate membrane filter (Sartorius stedim biotech, Goettingen, Germany) before filling the eluent organizer.

2.2. Ultra Performance Liquid Chromatography – Diode Array Detector (ULPC-DAD)

Ultra performance liquid chromatography (UPLC) was performed using a Waters Acquity H-class system (Waters Corporation, Milford, MA). Samples and standards were maintained at 4°C in the sampler manager prior to analyses. 5 µL of samples were then injected into an Acquity BEH C18 column (100 x 2.1 mm, 1.7 µm, Waters) thermostated at 45°C. Different compositions of mobile phases were evaluated to achieve the separation of quinapril and its degradation products. The general composition of the eluent consisted of an UPW/ACN mixture. Effects of the ratio UPW/ACN, pH and addition of buffering species (ammonium hydrogencarbonate, formic acid) on the chromatographic separation were evaluated. Flow rate was set at 0.4 mL min^{-1} and detection was made between 190 and 500 nm. Waters Empower™ chromatography software was used to control the chromatographic system and to record data.

89 **2.3. Ultra Performance Liquid Chromatography – Tandem Mass Spectrometry (ULPC-MS/MS)**

90 Liquid Chromatography Tandem mass spectrometry system consisted of an Acquity UPLC system (Waters
91 Corporation, Milford, MA) coupled with a triple quadrupole detector (Quattro premier, Micromass). 5 µL of
92 samples from the hydrolytic degradation experiments were separated on an Acquity BEH C18 column (100 x 2.1
93 mm, 1.7 µm, Waters). Isocratic separation was carried out with a mixture of eluent A (65%, vol.): eluent B (35%
94 vol.) at a flow rate of 0.4 mL min^{-1}. Eluent A consisted of an aqueous solution of ammonium hydrogencarbonate
95 (10 mM, pH 8.1); eluent B was ACN. Sampler manager and column oven were kept at 4°C and 45°C,
96 respectively. The MS analysis was performed by means of an electrospray ionization (ESI) interface both in
97 positive or negative ion mode with a capillary voltage of ± 3 kV. Indeed, according to the quinapril structure
98 both positive and negative modes could be applied, owing to the carboxylic and amide functional groups of
99 quinapril which could give protons during the ionization process on the one hand, and since both molecules
100 could be protonated on the other hand. In accordance with preliminary investigation as well as with literature
101 data [1-2, 31], a positive mode was chosen. Product ion spectra of quinapril and its degradation products were
102 acquired using N_2 as nebulizer and drying gas. The cone gas flow and the desolvation gas flow were set to 50 L
103 h^{-1} and 750 L h^{-1}, respectively. The source temperature and the desolvation gas temperature were 120 °C and 350
104 °C, respectively. The mass range (m/z) was 50-600.

105 **2.4. Stress study**

106 Stress studies were carried out under the conditions of heat and hydrolysis as mentioned in ICH Q1A (R2)
107 guideline [23]. Hydrolytic decomposition of quinapril was performed at 80 °C with 0.1 N HCl, water and 0.1 N
108 NaOH at an initial drug concentration of 0.5 mg mL^{-1} and 0.1 mg mL^{-1}. However, because quinapril was found
109 to be highly affected to alkaline degradation, studies were performed by reducing the concentration of sodium
110 hydroxide to 0.01 N. The approach suggested by Bakshi and Singh [32] was adopted for this study. A minimum
111 of four samples were generated for each stress condition; the blank solution stored under normal conditions, the
112 blank subjected to the same stress as the drug (quinapril), zero time sample containing the drug (which was
113 stored under normal conditions), and the drug solution subjected to stress treatment.

114 **2.5. Separation study and development of stability-indicating method**

115 UPLC-DAD experiments were performed on all reaction solutions individually, and then on a mixture of
116 degraded drug solutions. In order to obtain acceptable separation between quinapril and its degradation products,
117 as well as between the different degradation products, different logical modifications like change in pH, mobile
118 phase composition and column temperature adjustment were tested. To allow the transposition of the

chromatographic method from UPLC-DAD to UPLC-MS/MS simple rules should be respected; eluent composition must only involve volatile compounds to avoid salt deposit into the cone. In the first step, a mixture of UPW acidified at pH = 3 by formic acid and ACN was used as a mobile phase. Formic acid was selected because the apparent pKa of quinapril is equal to 5.7 and the conventional degradation pathway of ACEI leads to the formation of carboxylic acid by the ester function cleavage. However non-reproducible retention time and signals intensity were obtained with formic acid. Elution with ammonium hydrogencarbonate as the buffering compound led to more relevant results for the separation of quinapril from its degradation products; it also resulted in a retention time observed on the UPLC-DAD system comparable to that obtained in the UPLC-MS/MS system. The selected composition of the mobile phase consisted therefore of a mixture of 65 % (vol.) ammonium hydrogencarbonate 10 mM in UPW and 35 % (vol.) of ACN with a flow rate of 0.4 mL min^{-1}. From a practical standpoint, ammonium hydrogencarbonate is an ideal buffer for chromatographers since it provides excellent chromatographic behaviour and reproducible separation. Satisfactory results were obtained by the standard diode array and MS detection by using this buffer at a concentration of 10 mM. A major reason for using this concentration was to achieve maximum sensitivity of UV detection at low wavelengths. The detector was operated at 211 nm since this ACEI weakly absorbs in the UV region. This method was shown to provide fast and efficient separation of quinapril from its degradation products. In addition, hydrogencarbonate buffer is thermally decomposed in CO_2 and NH_3 in the MS interface since 60°C [33].

3. Results and discussion

3.1. Degradation behaviour of quinapril under thermal hydrolysis (alkaline, neutral and acidic conditions)

The estimation of the impurity profiles of bulk drug substances is one of the most important fields of activity in contemporary industrial pharmaceutical analysis. In general impurities present in excess of 0.1% should be identified and quantified by sufficiently selective methods, but drug registration authorities are increasingly interested by impurities in the range 0.01-0.1% for many reasons [21]. The guidance also indicates that degradation products that are not formed under accelerated or long term conditions do not need to be evaluated. Therefore, data from this study are typically used to evaluate quinapril stability. As shown in figure.1, all quinapril by-products detected under accelerated stability testing exceeded the identification thresholds in all stress conditions.

Quinapril stability under various conditions (alkaline, neutral and acidic) at 80°C was assessed as mentioned above and the results of the stability studies are collected in figure.1. All experiments were conducted at two concentration levels of quinapril (0.114 mM in alkaline medium and 1.14 mM in neutral and acidic media). The

149 results indicated that quinapril is degraded under the various considered conditions. Chromatographic analysis
150 showed that quinapril degradation occurred faster in alkaline medium compared to both acidic and neutral
151 media. Under alkaline conditions, quinapril degradation was observed from the first minutes yielding the
152 formation of two by-products eluted at a retention time of 0.55 and 0.66 min respectively. The chromatogram,
153 obtained on a C18 column (Figure 1.a), shows a very broad peak relative to the quinapril (RT= 1.35 min, ω=
154 0.48 min) and a peak splitting relative to the by-product eluted at 0.66 min. This chromatographic phenomenon
155 may be attributed to an equilibrium between the cis- and the trans-conformers that arose from the hindered
156 rotation around the amide bond having partial double bond character. It is consistent with studies showing that
157 similar chromatographic phenomenon has been observed with other ACEI such as enalapril [34], enalaprilat [35]
158 and lisinopril [36]. However, additional experiments by LC-DAD, which could be later considered, are needed to
159 confirm this assumption.
160 Acidic and neutral hydrolysis at 80°C led to the formation, within the first minutes, of a major by-product eluted
161 at 8.92 min. Other degradation products were also observed but after a significant contact time. The full scan
162 spectra were recorded for solutions obtained after 22 h of contact with 0.1 M HCl and water. As shown in fig. 1
163 the chromatographic method was able to resolve all the components in a mixture of stressed sample. The peaks
164 associated to degradation products were not only well-resolved from the drug, but also from one another. The
165 method thus proved to be selective and stability-indicating either for the study of acidic, neutral and alkaline
166 hydrolysis. On the other hand, reversed-phase UPLC conditions provided a general measure of the polarity of
167 each compound, useful for the interpretation of substructural differences between related compounds.
168 Comparison of chromatograms obtained under acidic and neutral conditions shows that quinapril present the
169 same UPLC profile in the two media (same retention time and same UV spectra). Nevertheless, the rate of
170 degradation under acidic hydrolysis is faster than under neutral hydrolysis. After a contact time of 23 hours
171 approximately 89 % of the initial amount of quinapril has been degraded under neutral conditions leading to the
172 formation of 5 separated by-products (Fig. 1.c). The UPLC profile obtained for the neutral degradation of
173 quinapril revealed that among these products, four are eluted within only one minute retention time, namely
174 before the starting material, indicating that these products are more polar than quinapril. These products
175 corresponded to 6% of the amount of degraded quinapril after 24 h contact time, whereas the major by-product
176 eluted at 8.92 min corresponded to 81% of that amount.

Under acidic conditions, 99 % of the drug was degraded after 24 h contact time at 80°C. Similarly to the neutral conditions, the by-product eluted at 8.92 min was the major product with an amount of 70% of the degraded quinapril, whereas the other by-products corresponded to only 9 % of the degraded drug.

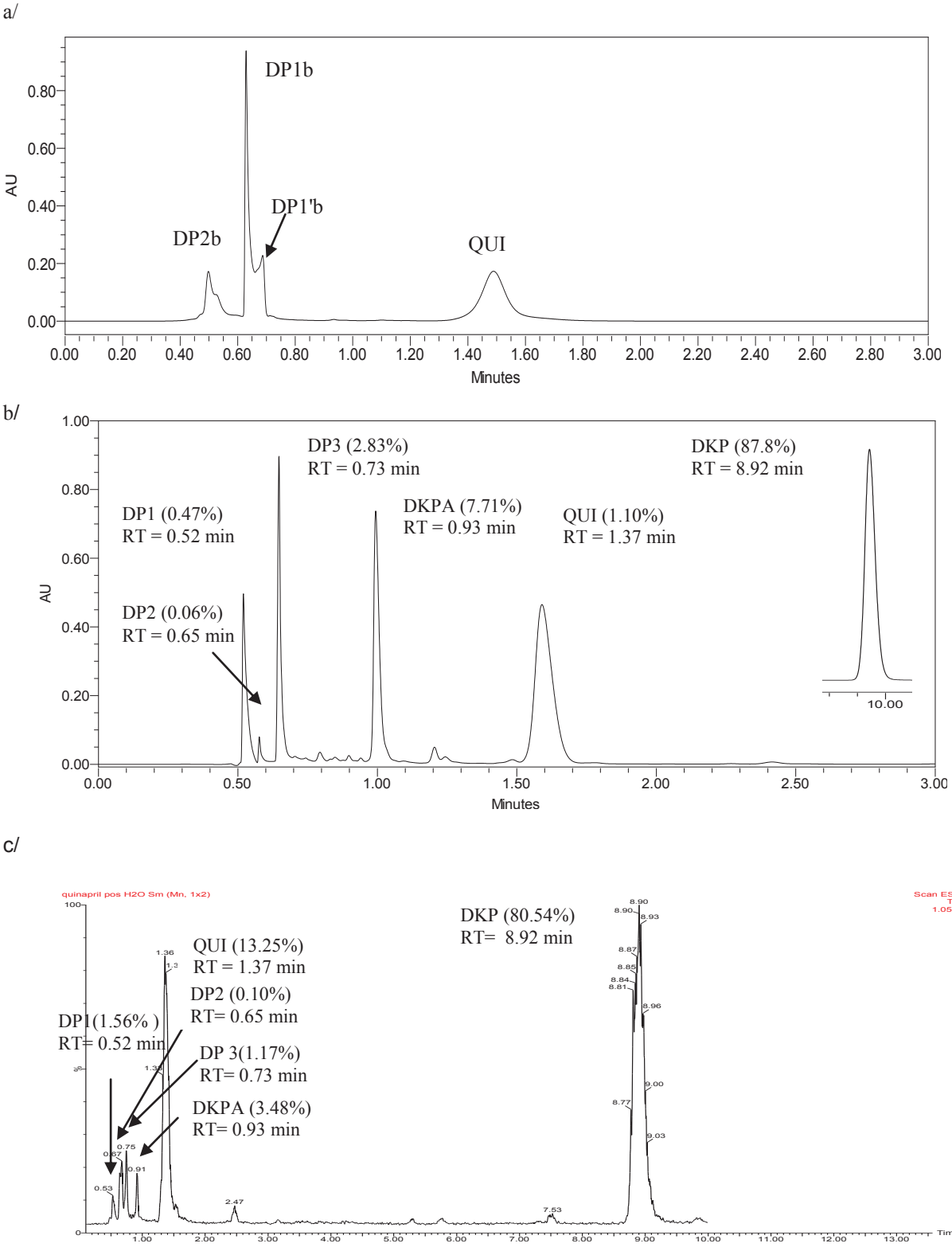


Fig. 1. Chromatograms of quinapril and its degradation products obtained under alkaline (a), acid (b) and neutral (c) conditions at T = 80 °C for 23 h contact time and an initial amount of 500 mg/L, using an UPLC/UV method on a BEH C18 column (1.7 µm, 2.1 mm × 100 mm) (Waters). Mobile phases: acetonitrile- ammonium hydrogencarbonate (pH = 8.2; 10 mM) -35:65, v/v). Flow rate: 0.4 mL/min. Wavelenght: 211 nm. Column temperature: 45°C.

Direct UV-vis spectrophotometric method shows that quinapril and its by-products present similar UV spectra with a benzenoid profile (Appendix A, Fig. A.1). These spectra are characterized by weak shoulders at high wavelength values (> 250 nm). It can be therefore concluded that this method was not suitable, neither for qualitative nor quantitative analysis of quinapril in a mixture of potential degradation products, since it caused a problem of interference. To overcome this problem, due to degradation products other than analytes derivative spectroscopy can be used as a qualitative and quantitative method [37-39]. On the other hand, this technique proved its ability for analysis of benzenoid drugs such as ACE-inhibitors [39] whose UV spectra exhibit a partial fine vibrational structure.

In order to investigate the ability of derivative spectrophotometric methods to measure the quinapril response in the presence of all the potential by-products, the derivative mode ^{1}D, ^{2}D, ^{3}D and ^{4}D (first, second, third and fourth-order derivative spectra), followed by currently used UPLC-DAD method were therefore plotted against wavelength. The degree of derivation was chosen in order to improve the resolution and the sensitivity of overlapping absorption and to find differences between UV spectra shapes of quinapril and its by-products. As can be seen from (Appendix A, fig.A.2), the derivation process allowed the conversion of large bands to sharp and intense peaks. Derivative UV spectra of quinapril and its by-products were quite similar and presented the same maximum of absorption except the degradation product eluted at 0.53 min. For instance, the second-order UV spectra of quinapril and its by-products exhibited intense peaks. As a result, derivative UV spectrophotometric method cannot be applied for quantitative or qualitative analysis of quinapril in the presence of its degradation products, due to the interference that can occur. In conclusion, the characteristic profile of the derivative spectra may not constitute a specific method useful to confirm drug identity and purity; no decrease in the amount of quinapril can be observed owing to its interference with degradation products.

In spite of the similarity of UV visible absorption and response factor of related compounds, their MS ionization efficiencies can be significantly different. The target degradation products under study have been therefore differentiated thereafter as DPx for acidic and neutral conditions and DPxb for alkaline medium.

3.2. Identification of degradation products of quinapril by UPLC-MS/MS

UPLC-MS/MS has become a powerful technique to determine drugs in various matrix, specific fragmentation pattern give selectivity and sensitivity and allow the accurate determination of numerous drugs such as ACEI [31][40]. The use of UPLC-MS/MS is particularly relevant for the elucidation of impurities and degradation product structures and to propose degradation mechanisms [41-43]. Last years, many studies have employed the LC-MS/MS technique in order to evaluate ACEI stability and to characterize their degradation products [42, 44-

45]. In order to elucidate the structure of degradation products induced by thermal hydrolysis process, LC-MS and LC-MS/MS substructural analysis methods have been therefore developed. These methods include information on molecular structures, chromatographic behaviour, molecular weight and MS/MS substructural information.

Using ammonium hydrogencarbonate as the mobile phase, the chromatographic method previously described was directly transferred from LC-DAD to LC-MS/MS. Due to its volatility, ammonium hydrogencarbonate is being an essential buffering specie for rapid LC-MS product identification [33]. However, formation of ammonium adducts could make more complex the MS/MS interpretation [40]. In a first time, full-scan UPLC-MS/MS of the degradation mixture ($[QUI]_0$ = 500 mg/L; [HCl] = 0.1 M; contact time = 23 h; T = 80°C) has been performed and showed that retention times observed for quinapril and the major degradation products were close to those obtained by LC-DAD.

MS spectra were acquired for each chromatographic peak. To further elucidate the structure of these degradation products, the MS/MS spectra (Appendix B, fig B.1 and fig B.2) of these products were acquired in an additional run with collision energy of 20 eV. This enabled to determine the elemental composition for the product ions of degradation products. The observed m/z values for molecular ion peak and major fragments of the quinapril and its degradation products under acidic and neutral media are listed in Table.1. These results prove that the same by-products were obtained under acidic and neutral conditions, with m/z values equal to m/z 252 (DP1), 178 (DP2), 280 (DP3), 393 (DKPA), and 421(DPK). It should be noted that ammonium adducts $[M+NH_4]^+$ was not observed.

229 **Table 1.** Observed m/z values for the $[M+H]^+$ ions and major fragments of quinapril and its by- products in

230 acidic, neutral and alkaline media.

Degradation products	Retention time (min)	$[M+H^+]$	Fragment ion	Fragment ion intensity (%)	Proposed elemental composition
DP1	0.53	252	206	100	$C_{12}H_{16}NO_2$
			160	19	$C_{11}H_{14}N$
			117	15	C_9H_9
			102	30	$C_4H_8NO_2$
			91	5	C_7H_7
DP2	0.68	178	132	100	$C_9H_{10}N$
DP3	0.74	280	234	50	$C_{14}H_{20}NO_2$
			206	100	$C_{12}H_{16}NO_2$
			160	13	$C_{10}H_{12}NO_2$
			130	10	$C_{11}H_{14}N$
			117	25	C_9H_9
			91	5	C_7H_7
DKPA	0.93	393	375	60	$C_{23}H_{23}N_2O_3$
			347	10	$C_{22}H_{23}N_2O_2$
			319	5	$C_{21}H_{23}N_2O$
			231	70	$C_{13}H_{14}N_2O_2$
			117	100	C_9H_9
			91	1	C_7H_7
Quinapril	1.37	439	365	8	$C_{22}H_{25}N_2O_3$
			234	78	$C_{14}H_{20}NO_2$
			206	2	$C_{12}H_{16}NO_2$
			160	4	$C_{11}H_{14}N$
			130	5	$C_6H_{12}NO_2$
			117	3	C_9H_9
			102	2	$C_4H_8NO_2$

DKP	8.92	421	375 347 319 231 117 91	95 13 5 100 50 2	$C_{23}H_{23}N_2O_3$ $C_{22}H_{23}N_2O_2$ $C_{21}H_{23}N_2O$ $C_{13}H_{14}N_2O_2$ C_9H_9 C_7H_7
PD2b	0.55	411	365 206 178 160 102	2.5 30 3.75 1 2	$C_{22}H_{25}N_2O_3$ $C_{12}H_{16}NO_2$ $C_{10}H_{13}NO_2$ $C_{11}H_{14}N$ $C_4H_8NO_2$
PD1b	0.67	411	365 206 178 160 102	2.5 31 4 0.5 1	$C_{12}H_{16}NO_2$ $C_{10}H_{13}NO_2$ $C_{11}H_{14}N$ $C_4H_8NO_2$ $C_4H_8NO_2$
PD1'b	0.76	411	365 206 178 160 102	2.5 32 2.5 1.25 1	$C_{22}H_{25}N_2O_3$ $C_{12}H_{16}NO_2$ $C_{10}H_{13}NO_2$ $C_{11}H_{14}N$ $C_4H_8NO_2$

In order to elucidate the structures of quinapril by-products, the first step was to understand the fragmentation pattern of the parent-drug substance. Indeed, the detailed mass spectrometry analysis of the fragmentation pattern of quinapril provides a basis for assessing structural assignment for the degradation products. As can be seen from Table 1, the fragmentation pattern for drug displayed the same behavior as reported by Burinsky and Sides [31] and Vikas shinde et al [2]. Indeed, the (+) ESI-MS/MS spectrum of quinapril, with a protonated molecular ion at m/z 439, showed a series of fragment ions of m/z values of 365, 234, 170, 160, 134, 130 and 117. The fragment ion with m/z 365, which was 74 Da less than the quinapril ion, resulted from the loss of the ester side chain ($OCOCH_2CH_3$). The most intense fragment at m/z 234 resulted from the cleavage of the bond

between the carbon atom of the amide group and the α-carbon, followed by further loss of CH_2=CH_2 to yield m/z 206 and subsequent elimination of styrene to produce m/z 102. Finally, the subsequent fragments at m/z 160, 134 and 117 originated from the aliphatic chain reduction from the product-ion m/z 206 (Fig. 2).

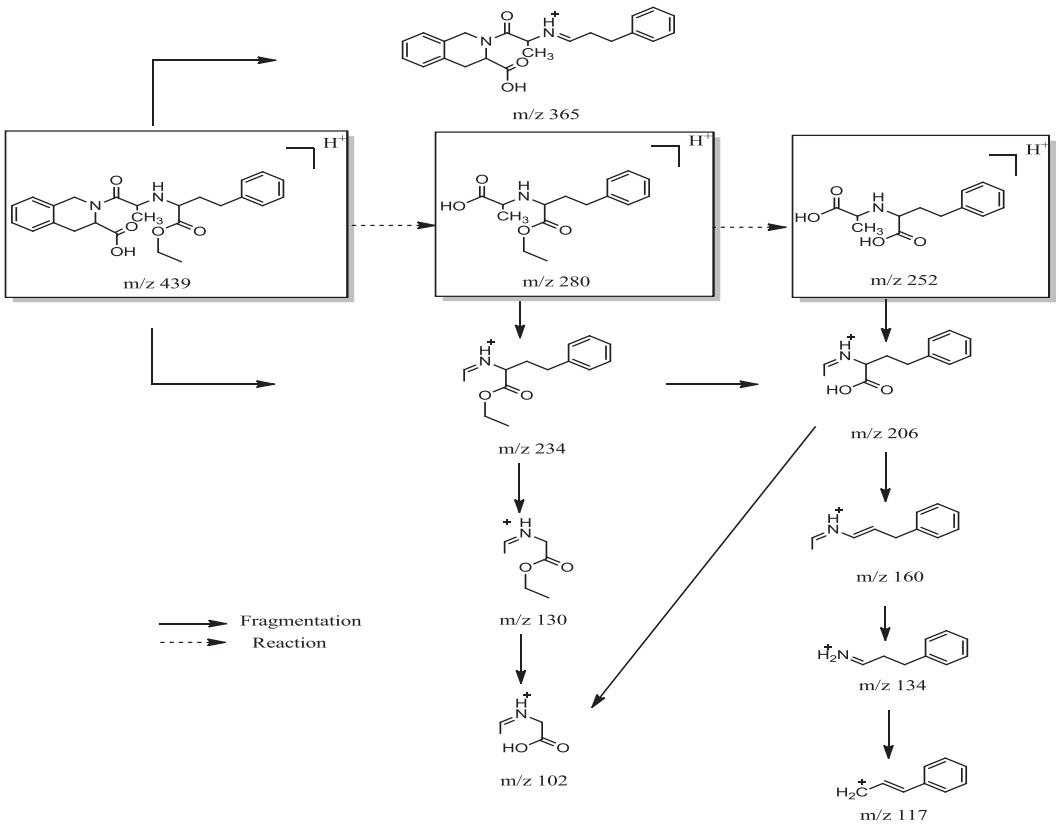


Fig. 2. Fragmentation pattern for quinapril (m/z 439) and some degradation products; DP3 (m/z 280) and DP1 (m/z 252)

According to the structure of quinapril, cyclization, ester and amide bond hydrolysis were the possible degradation pathway that can be foreseen. However, from Table 1 only two major degradation pathways of quinapril in acidic and neutral media were observed; cyclization was the main pathway, while hydrolysis of the amide bond was only observed at low levels in the samples.

The degradation product eluted at 8.92 min retention time, exhibited the same MS^1 spectrum with a molecular ion at m/z 421, both under acidic and neutral conditions. The MS^2 spectrum obtained by fragmentation of the m/z 421 ion led to a series of atypical ions (m/z 375, 347, 319, 231, 117, 91) (Fig.3) compared to quinapril fragmentation. With respect to the MS^2 spectrum (Appendix B, Fig. B.3), the mechanism of fragmentation presented in fig. 4 can be suggested from the diketopiperazine product (DKP). Formation of the diketopiperazine product requires deprotonation of the reacting amine followed by the addition of neutral nitrogen to the carbonyl of the neighboring carboxylic acid to form a tetrahedral intermediate, which then loses water to give the diketopiperazine product. Similar results were observed for some ACE inhibitors such as moexipril [46],

enalapril [47], lisinopril [48], ramipril [46, 49], xpril and perindopril [50] which yield to the diketopiperazine product under acidic and neutral conditions.

The compound eluted at a retention time of 0.93 min with a protonated molecular m/z 393 was 28 Da less than quinapril diketopiperazine. 28 Da corresponded to the loss of CH_2=CH_2 to yield to the diketopiperazine acid product ($C_{23}H_{24}N_2O_4$). This product characterized by the same fragment ions as quinapril diketopiperazine, can result from the hydrolysis of the ester function of DKP or cyclization of the diacid product with an m/z value of 411. However, the diacid product of quinapril was not detected both in neutral and acidic media. In order to confirm that the diacid product of quinapril with m/z value of 411 is not coeluted,a LCMS[1] attempts were made to follow the "LC/MS profile" of this compound (m/z=411). Results obtained proved that this product was not detected neither in acid medium nor neutral medium.

The last hypothesis was a direct transformation of the diacid product to yield DKPA. A simulation study could be later considered to confirm or deny this assumption.

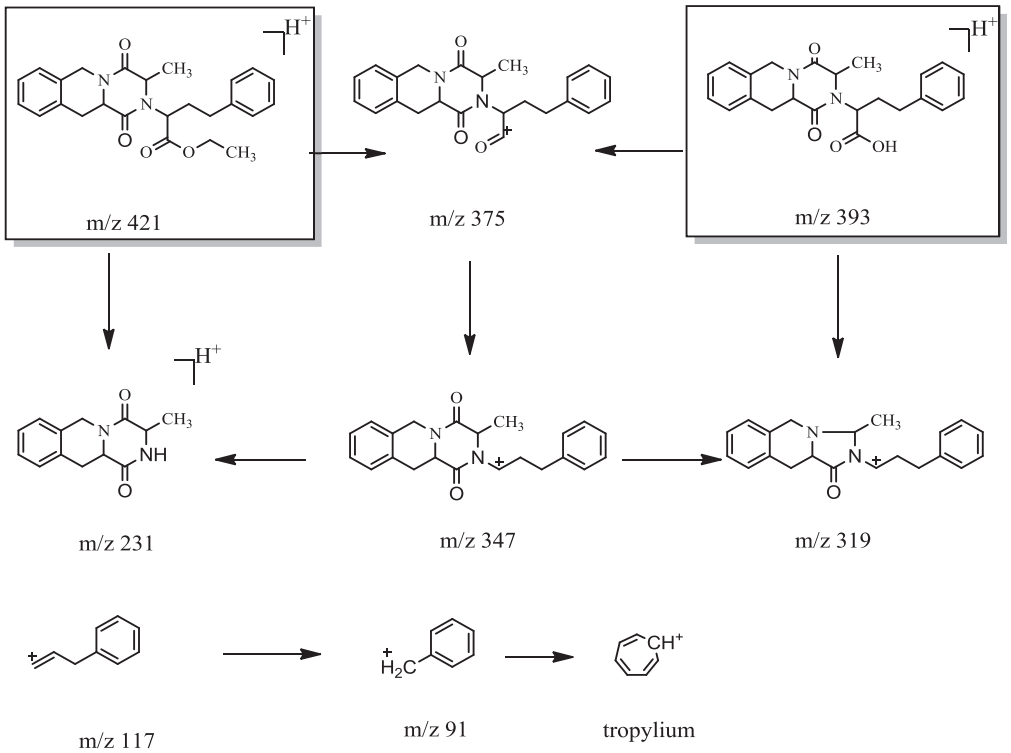


Fig. 3. Fragmentation pattern for degradation products DKP and DKPA with m/z 421 and 393, respectively

The second possible pathway of degradation, namely a hydrolysis of the amide bond, was confirmed by the presence of two degradation products DP2 and DP3 with protonated molecular [M+H^+], m/z 178 and 280, both

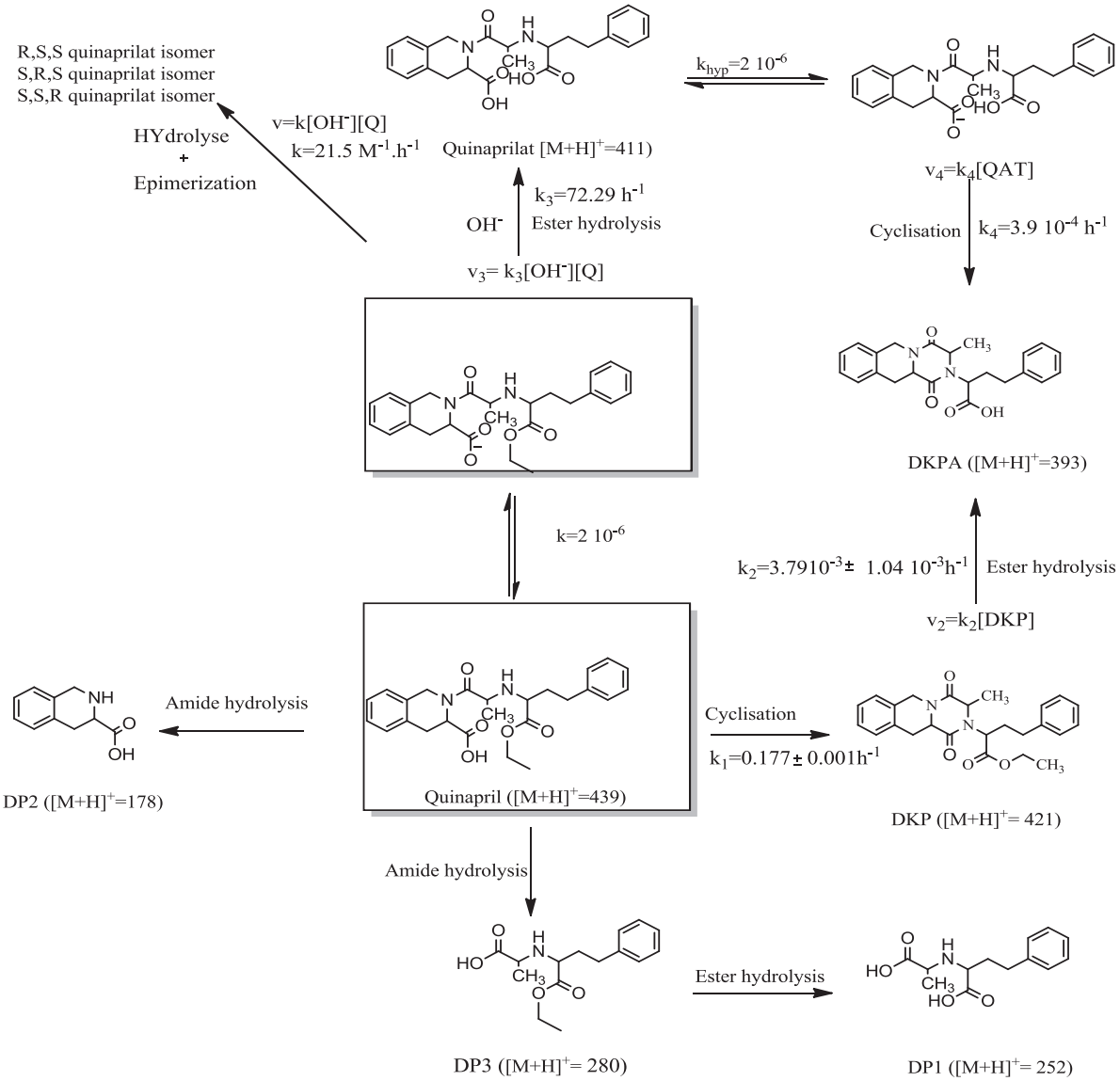
in neutral and acidic media. This result was in agreement with other findings [51-52], showing that the cleavage of the peptide bond is a common degradation pathway of peptides and proteins. Based on the MS^2 spectra (Appendix B, Fig. B.3), the elemental composition for these ions were $C_{10}H_{11}NO_2$ and $C_{15}H_{21}NO_4$ for m/z 178 and 280, respectively. The DP3 with an m/z value of 280 was characterized by a series of fragments 234, 206, 160, 130, 117, 91. The most intense fragment (m/z 206) corresponded to the loss of 74 Da relative to the ester function followed by further loss of formic acid (46 Da). Furthermore, the other fragments at m/z 130, 117and 91 were characteristics of intact part of the molecule ($C_9H_{12}N$). Except the fragment ion m/z 130, the by-product DP1, which differed from DP3 by 28 Da, displayed the same fragment ions characteristic of the intact part. 28 Da corresponded to a loss of an ethyl group generated by hydrolysis of an ester function. According to the elemental composition of this product, it can be assumed that this diacid product can be derived from the cleavage of the amide bond of the active metabolite quinaprilat or the hydrolysis of the ester function of the DP3 by-product with m/z 280 (fig.2).

These results seem to be in accordance with some studies showing that cyclization and amide bond hydrolysis occurred under acid and neutral conditions. In fact, it is well documented in the literature that dipeptides and proteins readily cyclize to diketopiperazine derivate and underwent a hydrolysis of the amide bond [51] [53]. The present work also shows that the rate of cyclization competes favorably with hydrolysis, in agreement with previous findings dealing with the forced degradation of some ACEI, namely fosinopril and enalapril maleate [52, 47]. These studies proved that the main decomposition route was the internal aminolysis reaction, producing the diketopiperazine and that the amide bond tends to cleave in acidic and neutral media. However, under these conditions ramipril and moexipril yield only the DKP product [48].

Because very low amounts of DP3 and DP4 were observed during the thermal hydrolysis, it is inferred that hydrolysis of this bond was slower than the formation of diketopiperazine. It is in agreement with earlier investigations showing that internal aminolysis of dipeptide methyl esters and amides to form diketopiperazine was much faster than the hydrolysis of ester and amide functionalities [53].

The exclusive formation of by-products with m/z 411 under alkaline conditions was in agreement with previous investigations which demonstrated that the ester function of enalapril maleate, ramipril and moexipril undergo hydrolysis to yield the dicarboxylic form of ACEI [47-48]. MS^2 analysis of these by-products shows the same ion fragments (365, 206, 178, 160,102). These products are relative to the quinaprilat and its isomers. The DP1b was characterized by a peak splitting, most likely due to the presence of the two conformers s-cis and s-trans around the amide bond N-C=O. Analysis by LC/MS and LC/DAD confirmed that the peak splitting was due to

304 the presence of two conformers. In fact, the purity of the peak relative to quinaprilat was checked by comparing
305 the UV spectra and by examination of the MS spectra. In addition, the effect of various operating conditions on
306 the retention peak, namely splitting and bond broadening of quinaprilat and quinapril, have been qualitatively
307 examined. This study provided more practical experimental conditions, allowing both the elution of quinaprilat
308 and quinapril as single peaks, while keeping at the same time an acceptable separation. The effect of various
309 factors on the conformational equilibrium s-cis/s-trans of quinapril and quinaprilat, namely the composition of
310 the mobile phase, flow rate, and column temperature was investigated and the results are shown in the supporting
311 information. The degradation product eluted at 0.55 min retention time shows the same fragmentation pattern as
312 quinaprilat and can result from the epimerization of the DP1; about 25% of the observed degradation in 0.01 N
313 NaOH was epimerized (Fig. 1.a). However, due to the lack of authentic isomers (RSS, SRR, SRS), the nature of
314 the epimer cannot be elucidated.
315 Similar results have been reported on moexiprilat degradation in 0.1 N KOH by Gu et al [54].
316 **3.3 Mechanism and kinetic of quinapril degradation**
317 Structural elucidation of by-products led to the proposal of the degradation mechanism given in Fig. 4. It is
318 postulated that cyclization is the major degradation process of quinapril under acidic and neutral conditions.
319 Formation of diketopiperazine is followed by the hydrolysis of the ester function to lead to the diketopiperazine
320 acid. Cleavage of the amide bond constitutes the second pathway of degradation of quinapril. In fact, the amide
321 bond undergoes hydrolysis to lead to the two by-products DP3 and DP2 with m/z 280 and 178 respectively. Thus
322 the DP1 by-product is generated from the hydrolysis of the ester function of DP3. Therefore, quinapril undergoes
323 hydrolysis of the ester function only under alkaline conditions.
324 In order to check the possibility of cyclization of quinaprilat to yield DKPA, two successive reactions were
325 performed. These reactions consisted of an alkaline hydrolysis (NaOH 0.01 N) of quinapril at 80 °C, until the
326 started material was exhausted, followed by an acidic hydrolysis (HCl 0.1N) at the same temperature. Analysis
327 of the chromatographic profile of quinaprilat under acidic conditions by LC/DAD and LC/MS shows that DKPA
328 was the major by-product formed. The kinetic study by the chromatographic method shows that the constant
329 rate of the formation of DKPA was $3.9\ 10^{-3}\ h^{-1}$.



330 **Fig. 4.** A proposed degradation pathway of quinapril under acidic (HCl (0.1N)), neutral and alkaline (NaOH
331 (0.01N)) conditions at 80°C.

332 To elucidate the pathways of quinapril decomposition under the studied conditions, the concentration-time
333 profiles of quinapril and major degradation products would be relevant. The kinetic of degradation of quinapril
334 was investigated under different conditions (acid, neutral and alkaline) and after exposure to heating at 80°C.
335 The remaining quinapril after various storage intervals was assessed by the stability-indicating UPLC method
336 mentioned above. At various contact times, 300 µL of solution was transferred into vials conserved in an ice bath
337 (-7°C) in order to stop the reaction. Samples were then immediately analysed by UPLC-DAD in order to
338 determine the remaining concentration of quinapril. However, in alkaline medium, the rate of quinapril
339 degradation was much faster than in neutral and acidic media. Quinapril reaction was followed for 2 hours in
340 alkaline medium, while it was followed for 24 hours in neutral and acidic media. Fig. 5 (Curves in point form)

shows a typical time course of disappearance of quinapril and formation of its major degradation products detected under the studied media. Main products were quinaprilat and its isomers products in alkaline medium, and both DKP and DKPA in acid and neutral media. Accordingly, cyclization of quinapril was much faster than hydrolysis of amide and ester bonds under acidic and neutral media.

As quinaprilat and quinaprilat isomers were only formed under basic medium, only the ester bond hydrolysis was considered. In all conditions, quinapril concentration decreased exponentially with time and since hydrochloric acid, water and sodium hydroxide are in large excess with respect to quinapril, the kinetics would be a typical pseudo-first-order process. This kinetic behaviour was compatible with earlier reports for other ACE inhibitors, such as enalapril maleate [50] [55], xpril, perindopril [50] [56], benazepril [57] and moexipril [12].

a/ b/ c/

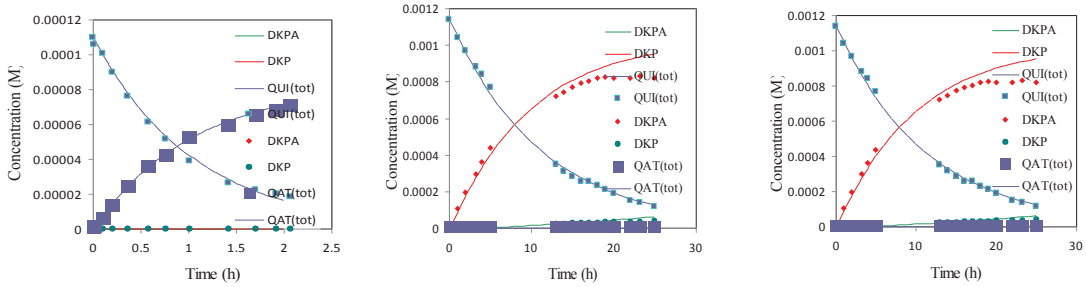


Fig. 5. Degradation of quinapril ([QUI]$_0$ = 500 ppm= 1.14 mM) at T = 80 °C under alkaline (a), acidic (b) and neutral (c) conditions. Experimental concentration-time profiles for quinapril, and diketopiperazine product (left axis) and degradation product (m/z 393) (right axis)

Fig.6 confirmed that at 80°C quinapril degraded faster under alkaline conditions compared to acidic and neutral conditions. The apparent first-order degradation rate constants at 80°C were calculated and found to be 0.19 h^{-1} ($t_{1/2}$= 3.70 h), 0.086 h^{-1} ($t_{1/2}$= 8.02 h) and 1.12 h^{-1} ($t_{1/2}$= 0.62 h) for acidic, neutral and alkaline degradation processes, respectively. These results proved that quinapril was more stable under neutral conditions, in agreement with previous works dealing with enalapril maleate [41], xpril and perindopril [37][44], and benazepril [50].

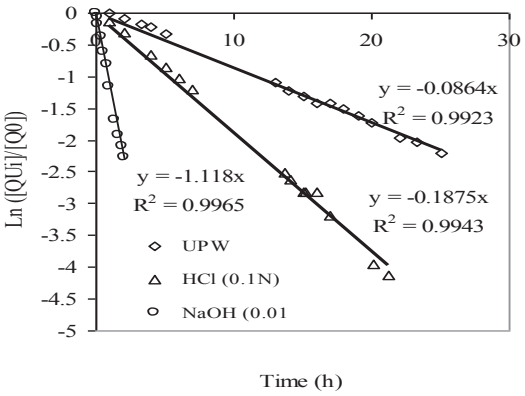


Fig. 6. First-order plots for the rate of disappearance of quinapril under alkaline, acidic ([HCl] = 0.1 M) and neutral conditions ($[QUI]_0$ = 500 ppm = 1.14 mM at 80°C).

3.5. Degradation pathway of quinapril

Kinetic model was developed according to the proposed degradation pathway of quinapril under all consideration media. Only the main product (quinaprilat, DKP and DKPA) were considered in the kinetic model. COPASI 4.8 [58] was used to calculate the rate constant from the experimental data point and to simulate the degradation curves of quinapril and the formation of DKP and DKPA. In order to fit the rate constants to experimental data, it was assumed that the molar extinction coefficient of quinapril and its by products were similar. Because cyclisation of quinapril or quinaprilat to form DKP or DKPA requires the protonation of the carboxylic formation, acid-base equilibria of quinapril and quinaprilat were considered. Rate constant were determined by the least square method using the evolutionary programming method for parameters optimization using COPASI software. Degradation rate constant of quinapril to form quinaprilat and quinaprilat isomers was determined by considering only the experiment under alkaline conditions. Other degradation rate constants (k_1, k_2) were evaluated with multiple experimental parameters (estimation formation considering experimental data point from acid and neutral conditions). Values and SD obtained for the kinetic rate constant were reported in fig. 4. A complete model including degradation pathway observed under all tested conditions was then developed and used to simulate the degradation curves of quinapril and the formation of the main by-products (Fig. 5). The proposed kinetic model allowed an accurate description of quinapril degradation.

Modelling the degradation curves to the equation rates that describe the reaction pathways proposed allowed to check the assumption given in fig.4. Curves (in continuous lines) under neutral and acidic media are given in fig. 5. The rate constants of disappearance of quinapril and the appearance of quinapril DKP, quinapril DKPA and quinaprilat were fitted. Pseudo-first-order kinetics were observed in all cases, and the rate constants (k_1 and k_2)

were determined and found to be 0.177±0.001 h^{-1}, 3.79 10^{-3}±1.04.10^{-3}, respectively. However quinaprilat was not formed, neither under acidic medium nor under neutral medium. So, k_1.[QH] was far superior to $k_3[Q^-]$ (QH and Q^- are respectively the protonated and unprotonated forms of quinapril respectively). Therefore, the DKPA formed during the degradation of quinapril must result from the hydrolysis of DKP rather than from the cyclization of quinaprilat. It was consistent with previous investigations demonstrating that ester and amide functional groups of dipeptides undergo intramolecular aminolysis at a much faster rate than hydrolysis [51].

Conclusion

The development of a stability-indicating UPLC assay method for quinapril allowed the separation of the drug and its by-products formed under various stress conditions.

Quinapril was found to be unstable in solution showing the formation of five degradation products. Structural elucidation performed by UPLC-MS-MS confirmed the presence of known by-products and allowed to propose the structure of unknown products. Comparison of the stability of quinapril under solution and solid states showed that kinetics and by-products distribution were altered.

Through detailed kinetic study and by-products distribution a complete model including degradation pathway observed under all tested conditions was developed.

References:

[1] Freed, A.L.; Kale, U.; Ando, H.; Rossi, D.T.; Kingsmill, C.A. Improving the detection of degradants and impurities in pharmaceutical drug products by applying massspectral and chromatographic searching, J. Pharm. Biomed. Anal. 2004, 35, 727–738.

[2] Shinde, V.; Trivedi, A.; Upadhayay, P.R.; Gupta, N.L.; Kanase, D.G.; Chikate, R.C. Degradation mechanism for a trace impurity in quinapril drug by tandem mass and precursor ions studies, J. Mass Spec. 2007, 21 3156-3160.

[3] Cŭdina, O.; Janković, I.; Cômor, M.; Vladimirov, S. Interaction of quinapril anion with cationic surfactant micelles of cetyltrimethylammonium bromide, J. Colloid Interface Sci. 2006, 301, 692-696.

[4] Abbara, Ch.; Aymard, G.; Hinh, S.; Diquet, B. Simultaneous determination of quinapril and its active metabolite quinaprilat in human plasma using high-performance liquid chromatography with ultraviolet detection, J. Chromat B. 2002, 766, 199-207.

[5] Hillaert, S.; De Grauwe, K.; Van den Bossche, W. Simultaneous determination of hydrochlorothiazide and several inhibitors of angiotensin-converting enzyme by capillary electrophoresis, J. Chromat A. 2001,924, 439-449.

406 [6] Mc Areavey, D.; Robertson, J.I. Angiotensin converting enzyme inhibitors and moderate hypertension,
407 Drugs. 1990, 40, 326-345.
408 [7] Makwana, K.; Dhamecha, R.V.; Pandya, N. A rugged and economic method for the estimation of quinapril
409 and its metabolite in human serum by LC/MS/MS detection for clinical trials, Int. J. Pharm. Pharm. Sci. 2011, 3,
410 112-116.
411 [8] Kieback, A.G.; Felix, S.B.; Reffelmann,T. Quinaprilat: a review of its pharmacokinetics, pharmacodynamics,
412 toxicological data and clinical application, Expert Opinion on Drug Metabolism & Toxicology. 2009, 5, 1337-
413 1347.
414 [9] Haefeli, W.E.; Linder, L.; Lüscher, T.F. Quinaprilat Induces Arterial Vasodilation Mediated by Nitric Oxide
415 in Humans, J. Hypertension. 1997, 30, 912-917.
416 [10] Stanisz, B. Kinetics of lisinopril degradation in solid phase, React.Kinet.Catal.Lett. 2005, 85, 145-152.
417 [11] Gu, L.; Strickley, R.G.; Chi, L.H.; Chowhan, Z.T. Drug- excipient incompatibility studies of the dipeptide
418 angiotensin converting enzyme inhibitor, moexipril hydrochloride dry powder vs. wet granulation, Pharm. Res.
419 1990, 7, 379-383.
420 [12] Strickley, R.G.; Visor, G.C.; Lin, L.; Gu, L. An unexpected pH effect on the stability of moexipril
421 lyophilized powder, Pharm. Res. 1989, 6, 971-975.
422 [13] Byrn, S. R.; Xu, W.; Newman, A.W. Chemical reactivity in solid-state pharmaceuticals: formulation
423 implications, Adv. Drug Deliver. Rev. 2001, 48, 115-136.
424 [14] Al-Omari, M.M.; Abdelah, M.K.; Badwan, A.A.; Jaber, A.M.Y. Effect of the drug-matrix on the stability of
425 enalapril maleate in tablet formulations, J. Pharm. Biomed. Anal. 2001, 25, 893-902.
426 [15] Stanisz, B.; Liquid Chromatographic Studies of the Stability of Benazepril in Pure Form and in Tablets, J.
427 Liq. Chromatogr. Related Technol. 2005, 27, 3103-3119.
428 [16] Shetty, S.K.; Surendranath, K.V.; Radhakrishnanand, P.; Borkar, R.M.; Devrukhakar, P.S.; Jogul, J.;
429 Tripathi, U.M. Stress Degradation Behavior of a Polypill and Development of Stability Indicating UHPLC
430 Method for the Simultaneous Estimation of Aspirin, Atorvastatin, Ramipril and Metoprolol Succinate, Am. J.
431 Anal. Chem. 2011, 2, 401-410.
432 [17] Stanisz, B. The stability of quinapril hydrochloride-a mixture of amorphous and crystalline forms (QHCl-
433 AC) in solid phase, Acta Pol Pharm. 2003, 60, 443-449.
434 [18] Stanisz, B.; The influence of pharmaceutical excipients on quinapril hydrochloride stability, Acta Pol.
435 Pharm. 2005, 62, 189-193.

436 [19] Shumet, A.H.; Bogner, R. H. Solid-state surface acidity and pH-stability profiles of amorphous quinapril
437 hydrochloride and silicate formulations, J. Pharm. Sci. 2010, 99, 2786-2799.
438 [20] Waterman, K.C.; Adami, R.C. Accelearated aging: prediction of chemical stability of pharmaceuticals, Int.
439 J. Pharm. 2005, 293, 101-125.
440 [21] Gororg, S.; Babjak, M.; Balogh, G.; Brlik, J.; Csehi, A.; Dravecz, F.; Gazdag, M.; Horvath, P.; Lauko, A.;
441 Varga, K.; Drug impurity profiling strategies, Talanta. 1997, 44, 1517-1526.
442 [22] Gorog, S. New safe medicines faster: the role of analytical chemistry, TrAC Trends Anal. Chem. 2003, 22,
443 407-415.
444 [23] ICH In International Conference on Harmonisation of Technical Requirements for Registration of
445 Pharmaceuticals for Human Use. Stability testing of new drug substances and products Q1A(R2). 2003.
446 [24] Bonazzi, D.; Gotti, R.; Andrisano, V.; Cavrini, V. Analysis of ACE inhibitors in pharmaceutical dosage
447 forms by derivative UV spectroscopy and liquid chromatography (HPLC), J. Pharm. Biomed. Anal. 1997, 16,
448 431-438.
449 [25] Hillaerta, S.; Vander Heydenb, Y.; Van den Bossche, W. Optimisation by experimental design of a capillary
450 electrophoretic method for the separation of several inhibitors of angiotensin converting enzyme using
451 alkylsulphonates, J. Chromat A. 2002, 978, 231-242
452 [26] Kugler, A.R.; Olson, S.C.; Smith, D.E. Determination of quinapril and quinaprilat by high performance
453 liquid chromatography with radio chemical detection, coupled to liquid scintillation counting spectrometry, J.
454 Chromat B. 1995, 666, 360-367.
455 [27] Lu, C.Y., Liu, F.T.; Feng, C.H. Quantitation of quinapril in human plasma by matrix-assisted laser
456 desorption ionization time-of-flight mass spectrometry with quinolone matrix additives, J. Chromat B. 2011,
457 879, 2688- 2694.
458 [28] Dasandi, B.; Shah, S.; Shivprakash, Determination of quinapril and quinaprilat in human plasma by
459 ultraperformance liquid chromatography–electrospray ionization mass spectrometry, Biomed. Chromatogr.
460 2009, 23, 492-498.
461 [29] Prieto, J.A.; Jiménez, R.M.; Alonso, R.M. Square wave voltammetric determination of the angiotensin
462 converting enzyme inhibitors cilazapril, quinapril and ramipril in pharmaceutical formulations, Il Farmaco.
463 2003, 58, 343-350.

464 [30] Vovk, I.; Popović, G.; Simonovska, B.; Albreht, A.; Agbaba, D. Ultra-thin-layer chromatography mass
465 spectrometry and thin-layer chromatography mass spectrometry of single peptides of angiotensin-converting
466 enzyme inhibitors, J. Chromat A. 2011, 1218, 3089-3094.
467 [31] Burinsky, D.; Sides, S.L. Mass spectral fragmentation reactions of angiotensin-converting enzyme (ACE)
468 inhibitors, J. Am. Soc.Mass Spec. 2004, 15, 1300-1314
469 [32] Bakshi, M.; Singh, S. Development of validated stability-indicating assay methods-
470 critical review, J. Pharm. Biomed. Anal. 2002, 28, 1011-1040.
471 [33] Espada, A.; Rivera-Sagredo, A. Ammonium hydrogencarbonate, an excellent buffer for the analysis of basic
472 drugs by liquid chromatography–mass spectrometry at high pH, J. Chromat A. 2003, 987, 211-220.
473 [34] Trabelsi, H.; Bouabdallah, S.; Sabbah, S.; Raouafi, F.; Bouzouita, K. Study of the cis–trans isomerization of
474 enalapril by reversed-phase liquid chromatography, J. Chromat A. 2000, 871, 189-199.
475 [35] Bouabdallah, S.; Trabelsi, H.; Ben Dhia, T.; Sabbah, S.; Bouzouita, K.; Khaddar, R. RP-HPLC and NMR
476 study of cis–trans isomerization of enalaprilat, J. Pharm. Biomed. Anal. 2003, 31, 731-741.
477 [36] Bouabdallah, S.; Trabelsi, H.; Bouzouita, K.; Sabbah, S. Reversed-phase liquid chromatography
478 of lisinopril conformers, J. Biochem. Bioph. Methods. 2002, 54, 391-405.
479 [37] Lagesson, V.; Lagesson-Andrasko, L.; Andrasko, J.; Baco, F. Identification of compounds and specific
480 functional groups in the wavelength region 168-330 nm using gas chromatography with UV detection, J.
481 Chromat A. 2000, 867, 187-206.
482 [38] Sanchez Rojas, F.; Bosch Ojeda, C. Recent development in derivative ultraviolet/visible absorption
483 spectrophotometry: 2004-2008, Analyt. Chim. Acta. 2009, 635, 22-44.
484 [39] Bonazzi, D.; Gotti, R.; Andrisano, V.; Cavrini, V. Analysis of ACE inhibitors in pharmaceutical dosage
485 forms by derivative UV spectroscopy and liquid chromatography (HPLC), J. Pharm. Biomed. Anal. 1997, 16,
486 431-438.
487 [40] Niessen, W. M. A. Fragmentation of toxicologically relevant drugs in positive-ion liquid chromatography–
488 tandem mass spectrometry, Mass Spec. Rev. 2011, 30, 626-663.
489 [41] Rourick, R. A.; Volk, K. J.; Klohr, S. E.; Spears, T.; Kerns, E. H.; Lee, M. S. Predictive strategy for the
490 rapid structure elucidation of drug degradants, J. Pharm. Biomed. Anal. 1996, 14,1743-1752.
491 [42] Marín, A.; Barbas, C. LC/MS for the degradation profiling of cough–cold products under forced conditions,
492 J. Pharm. Biomed. Anal. 2004, 35, 1035-1045.

493 [43] Gentili, A.; Marchese, S.; Perret, D. MS techniques for analyzing phenols, their metabolites and
494 transformation products of environmental interest, TrAC Trends Anal. Chem. 2008, 27, 888-903.
495 [44] Pérez, S.; Eichhorn, P.; Barceló, D.; Structural Characterization of Photodegradation Products of Enalapril
496 and Its Metabolite Enalaprilat Obtained under Simulated Environmental Conditions by Hybrid Quadrupole-
497 Linear Ion Trap-MS and Quadrupole-Time-of-Flight-MS, Anal. Chem. 2007, 79, 8293-8300.
498 [45] Bhardwaj, S.P.; Singh, S. Study of forced degradation behavior of enalapril maleate by LC and LC–MS and
499 development of a validated stability-indicating assay method, J. Pharm. Biomed. Anal. 2008, 46, 113-120.
500 [46] Elshanawane, A.; Mostafa, S.; Elgawish, M. Application of a Validated, Stability-Indicating LC Method to
501 Stress Degradation Studies of Ramipril and Moexipril.HCl, Chromatographia. 2008, 67, 567-573.
502 [47] Lima, D.M.; dos Santos, L.D.; Lima, E.M. Stability and in vitro release profile of enalapril maleate from
503 different commercially available tablets: Possible therapeutic implications, J. Pharm. Biomed. Anal. 2008, 47,
504 934-937.
505 [48] Beasley, C.A.; Shaw, J.; Zhao, Z.; Reed, R.A. Development and validation of a stability indicating HPLC
506 method for determination of lisinopril, lisinopril degradation product and parabens in the lisinopril
507 extemporaneous formulation, J. Pharm. Biomed. Anal. 2005, 37, 559-567.
508 [49] Hanyšová, L.; Václavková, M.; Dohnal, J.; Klimeš, J. Stability of ramipril in the solvents of different pH, J.
509 Pharm. Biomed. Anal. 2005, 37, 1179-1183.
510 [50] Roškar, R.; Simončič, Z.; Gartner, A.; Kmetec, V. Stability of new potential ACE inhibitor in the aqueous
511 solutions of different pH, J. Pharm. Biomed. Anal. 2009, 49, 295-303.
512 [51] Goolcharran, Ch.; Borchardt, R.T.; Kinetic of Diketopiperazine Formation Using Model Peptides, J.
513 Pharm. Sci. 1998, 87, 283-288.
514 [52] Radzicka, A.; Wolfenden, R. Rates of Uncatalysed Peptide Bond Hydrolysis in Neutral solution and the
515 Transition State Affinities of Proteases, J. Am. Chem. Soc. 1996, 118, 6105-6109.
516 [53] Jančić, B.; Medenica, M.; Ivanović, D.; Janković, S.; Malenović, A. Monitoring of fosinopril sodium
517 impurities by liquid chromatography–mass spectrometry including the neural networks in method evaluation, J.
518 Chromat A. 2008, 1189, 366-373.
519 [54] Simončič, Z.; Zupančič, P.; Roškar, R.; Gartner, A.; Kogej, K.; Kmetec, V.; Use of microcalorimetry in
520 determination of stability of enalapril maleate and enalapril maleate tablet formulations, Int. J. Pharm. 2007, 342,
521 145-151.

522 [55] Simoncic, Z.; Rokar, R.; Gartner, A.; Kogej, K.; Kmetec, V.; The use of microcalorimetry and HPLC for
523 the determination of degradation kinetics and thermodynamic parameters of Perindopril Erbumine in aqueous
524 solutions, Int. J. Pharm. 2008, 356, 200-205.
525 [56] Gana, M.; Panderi, I.; Parissi-Poulou, M.; Tsantili-Kakoulidou, A. Kinetics of the acidic and enzymatic
526 hydrolysis of benazepril HCl studied by LC. J. Pharm. Biomed. Anal. 2002, 27, 107-116.
527 [57] Gu, L.; Robert G.Strickley, A. Preformulation stability studies of the new Dipeptide Angiotensin-
528 Converting Enzyme Inhibitor RS-10029, Pharm. Res. 1988, 5, 765-771.
529 [58] Hoops, S.; Sahle, S.; Gauges, R.; Lee, C.; Pahle, J.; Simus, N.; Singhal, M.; Xu, L.; Mendes, P.; Kummer,
530 V. COPASI – a complex Pathway simulator. Bioinformatics. 2006, 22, 3067-74.